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Modulation of Pathogenic B Cells through Inhibition of Phosphatidylinositol 3-Kinases

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Note: An abstract is required to be provided in Block 14

This proposal will address the FY12 PRMRP topic area on lupus. Lupus is a life threatening disease that primarily affects women. Lupus patients develop antibodies that recognize proteins made by the body. This leads to tissue damage and complexes of the antibodies bound to the proteins can lodge in the kidneys resulting in damage to the filtering capacity of the kidney. The disease is most often managed using drugs that nonspecifically reduce inflammation and suppress the immune system. However that leaves the patient susceptible to other types of infections. Lupus treatment could be improved by specifically targeting the B cells involved in making the "self" antibodies. This proposal outlines one possible approach that could help solve this problem.

B cells express an important signaling molecule called PI3 kinase (PI3K). Activation of this enzyme leads to induction of survival pathways and is needed to promote development of antibodies. Therefore inhibition of PI3 kinase is expected to be beneficial for lupus by impairing survival of the pathogenic B cells and inhibiting their ability to produce antibodies. B cells express a specific form of PI3 kinase called delta. Small molecule inhibitors of the delta isoform have been shown to specifically kill B cell malignancies but leave other cells unaffected. Based on the success of the delta inhibitor in cancer research, it is anticipated that this approach will be particularly useful in lupus. Mice that are genetically predisposed to developing lupus will be treated with the PI3K delta inhibitor to determine if this ameliorates disease. If this works, it will provide an unprecedented level of control to target B cells and affect them by two mechanisms—survival and antibody production.

B cells also have survival mechanisms that work independently of PI3 kinase. One new lupus treatment is based on interfering with the other survival pathways. The drug, Benlysta, specifically neutralizes a B cell survival factor called BAFF (also known as Blys), which causes death of the mature antibody secreting B cells. Unfortunately it does not work for everyone and has shown little help among African-Americans. One possible reason is that the survival pathways mediated by PI3 kinase are capable to keeping many of the antibody producing B cells alive. Therefore, cultured B cells will be treated under conditions that mimic their interactions in the body to determine if interfering with both PI3K dependent and BAFF dependent survival results in even more B cell death than either approach alone. If true, experiments to test this in lupus prone animal models will be performed in the future.

Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

   This project focuses on use of novel PI3kinase inhibitors to treat lupus. The PI3K/Akt pathway is a highly conserved pathway involved in numerous processes including survival. The immune system expresses a novel PI3K isoform referred to as delta, which plays a critical role in B cell signaling, antibody production, and survival. Recently PI3Kδ inhibitors have been developed to treat B cell malignancies. Since they target tumors that often have characteristics similar to antibody secreting B cells, we reasoned that a similar approach may be useful for treating lupus, a disease resulting in production of antibodies recognizes "self" components, such as nuclear proteins and DNA. These antibodies can cause additional damage because immune complexes lodge in the kidney which results in further damage. These experiments will test the hypothesis that PI3Kδ inhibition will reduce the frequency of antibody secreting B cells in a mouse model for lupus, which results in less kidney damage and increased lifespan.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).
   Lupus, PI3K, B cell, signal transduction

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Progress Report for PI3K inhibitor on Lupus

Aim 1. Evaluate whether inhibition of PI3Kδ ameloriates the clinical pathology of lupus in NZB/NZW F1 mice

Aim 1. Our initial animal studies were designed to begin assessing the impact of treating diseased mice with CAL101, a PI3Kδ inhibitor. Previous work by others showed that inhibition of PI3Kδ was a promising treatment for some classes of B cell malignancies. In fact, this class of inhibitors has been recently approved for mantle cell lymphoma. Tonic B cell receptor (BCR) signaling is needed for B cell survival and BCR signaling in general leads to antibody and cytokine production. While B cells express multiple isoforms of PI3K, BCR signaling is highly dependent on delta isoform. We reasoned that inhibitors of PI3Kδ would lead to reduced

antibody production and should decrease the number of activated B cells. This would be expected to reduce the amount of disease in lupus.

Using the NZB/NZW lupus mouse model, we first established when we could initially detect anti-dsDNA antibodies, one of the pathogenic classes of antibodies found in lupus. Once they were readily detectable we began to dose mice with the drug to determine how that affected different B cell populations. In our hands, we had to wait until the mice were 8 months old before we obtained high levels of pathogenic antibody. The mice were then dosed 2x daily with 10 mg/kg CAL101 (idelalisib), a PI3Kδ inhibitor that was recently FDA approved. After one month of dosing, a cohort of mice were euthanized and spleens, blood, bone marrow, and kidneys isolated for further analysis.

Flow cytometry was performed on spleens and analyzed for presence of Tfh cells and germinal center (GC) B cells. These B cells are undergoing affinity maturation in response to antigen. The Tfh cells are a specialized class of T cells that help drive the germinal center response. In a naïve mouse, both Tfh and GC B cells are rare. In lupus models they can be abundant. Note in the figure below that Tfh comprise ~40% of the splenic T cell population. For comparison, an immunized mouse would contain only ~1% Tfh, emphasizing the abundance of this critical population in this lupus model. Drug treatment reduces the frequency of Tfh ~4-fold. Similarly GC B cells are elevated in the spleens of lupus mice and treatment with PI3Kδ inhibitor reduces them to near background levels. To confirm these results, we have spleens from vehicle and inhibitor treated mice that will be analyzed by immunofluorescence to determine if the frequency of germinal centers decreases. We also analyzed B cell subsets to determine if the PI3Kδ inhibitor generally ablates all B cells or if select subsets are affected. As shown in the figure below, the class-switched B cells are markedly decreased by drug, whereas the global number of mature B cells is unaffected. There appears to be a modest effect on the immature class of B cells. Because class switched B cells decreases following PI3Kd inhibition, we also established the ratio of T:B cells. This provides a more global view for ascertaining whether the drug affects B cells. Analysis of spleen, blood, and bone marrow shows that the ratio is not demonstrably altered by the drug.

We collected serum from the mice but have not yet analyzed it for the levels of anti-dsDNA antibodies or total IgG. This will be performed during the latter part of the funding period. In addition we have kidneys frozen for cryosectioning to determine if immunoglobulin deposits are reduced after drug treatment. We are analyzing a new cohort of mice to determine if treatment with CAL101 extends the life of the mice.

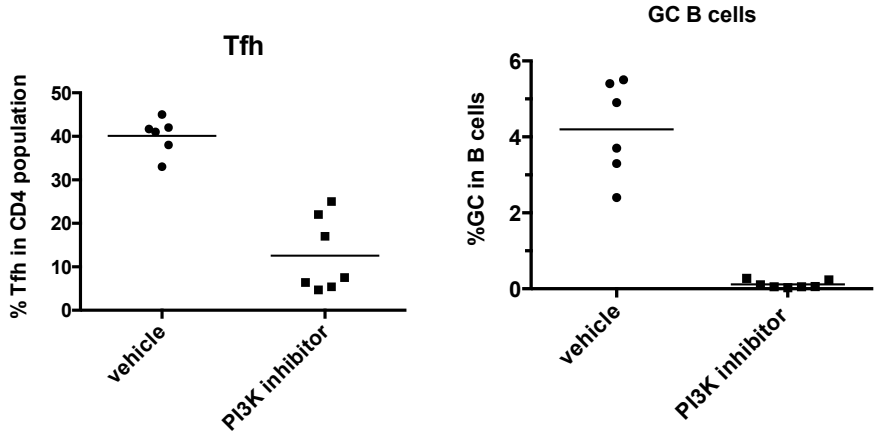

Fig 1. PI3Kδ inhibition decreases Tfh and GC B cells in lupus mice. Mice were treated for 1 month, then spleens analyzed by flow cytometry. Results are summarized in the graphs.

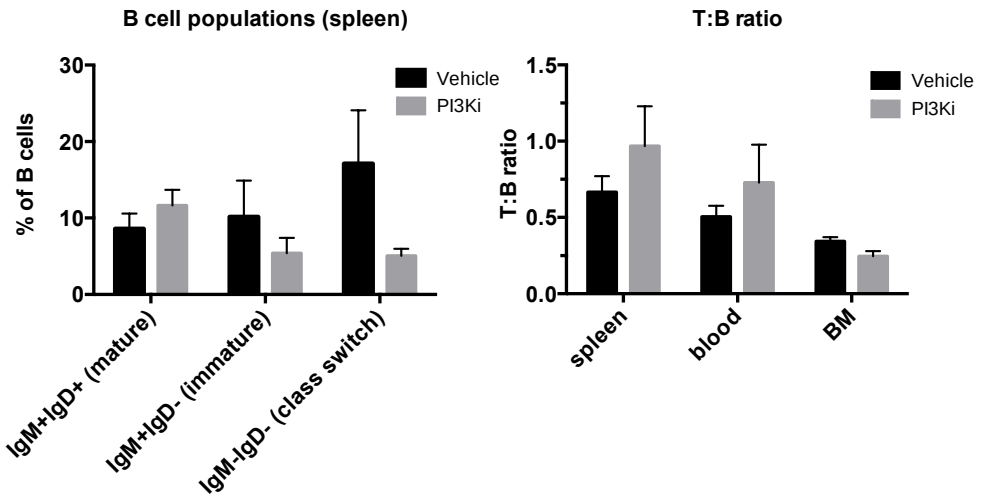

Fig 2. PI3Kδ inhibition disproportionately affects class switched B cells. Total B cells from spleen, blood, or bone marrow were analyzed. The drug appears to reduce class switched B cells, the population that is expected to produce pathogenic antibodies. Significantly, the drug does not cause wholesale loss of B cell as shown by the T:B ratio.

Aim 2. Determine if simultaneous inhibition of PI3K and BAFF synergistically impairs survival of pathogenic B cells.

Task 1. Purify B cells to determine optimal dose of BAFF and CAL101 (PI3Kδ inhibitor).
Task 2. B cell proliferation assays.
Task 3. B cell proliferation assays under suboptimal conditions

Aim 2. While waiting until the mice developed anti-dsDNA antibodies, we used some animals to isolate splenic B cells and activate them in culture to determine if PI3Kδ inhibition would alter B cell proliferation. Lupus patients have higher levels of circulating BAFF, a B cell survival factor. It has been hypothesized that the combination of BAFF and chronic B cell activation maintains B cells and they do not undergo apoptosis are readily, so the circulating pool of pathogenic B cells stays elevated. This has lead to a new treatment using anti-BAFF. Although not everyone improves with this treatment, it shows some evidence of efficacy in select populations. Since BCR induced survival/proliferation relies heavily on PI3Kδ activity, we reasoned that inhibiting this isoform would reduce proliferation. However, since BAFF is elevated in lupus patients, it was unclear whether it could confer a survival advantage in the presence of the PI3Kδ inhibitor. To begin addressing this, we performed in vitro cultures where we activated B cells through the BCR in the presence/absence of BAFF and CAL 101, a PI3Kδ inhibitor. Although CAL101 has been used to block in vitro proliferation, those reports used 1 uM of drug, which we found to be toxic. We were able to use as little as 10 nM CAL101 and obtain clear inhibition of proliferation. The BCR was activated by cross-linking with anti-IgM. Inclusion of 5 ng/ml BAFF increased overall proliferation ~3-fold, probably by preventing apoptosis. The presence of CAL101 blocked nearly all the proliferation induced by anti-IgM, whereas BAFF was able to only marginally improve proliferation. These results suggest that inhibiting PI3Kδ reduces B cell proliferation and probably survival, even in the presence of

BAFF. Furthermore it lends credence to concept that PI3Kδ blockade may be a viable therapeutic option for lupus. Although beyond the scope of this grant, it would be of clinical interest to combine CAL101 with anti-BAFF treatment. If there is synergy, it may be possible to have lupus patients receive intermittent treatments rather than being chronically exposed to these drugs.

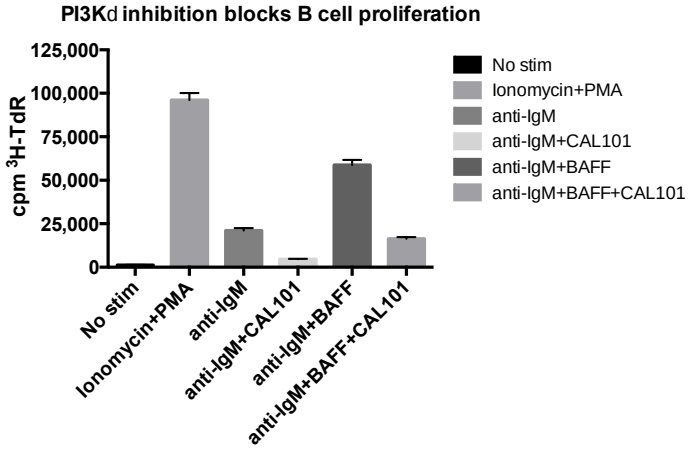

Fig 3. PI3Kδ inhibition reduces BCR induced proliferation. Purified B cells were plated in triplicate in microtiter dishes in the presence of 100 μg/ml anti-IgM and 5 ng/ml BAFF. After 3 days, 1μCi ^{3}H-thymidine was added and cells harvested the next morning. Ionomycin/PMA was used as a positive control. Note that CAL101 markedly reduces proliferation.

During the last phase of this grant we will perform additional experiments to examine if there is a more pronounced effect of PI3K inhibition under suboptimal activation conditions as this may more accurately reflect the normal in vivo environment.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

This is an annual progress report. A final progress report will be issued when the project is completed

- Initial in vitro experiments demonstrated that PI3K inhibition blocked B cell proliferation induced by activation of the B cell antigen receptor. Inclusion of the BAFF survival factor did not markedly improve on proliferation when the inhibitor was present.
- NZB/NZW mice (lupus model) were maintained to determine the age when anti-dsDNA antibodies were readily detectable. We found that the mice needed to be ~8 months old.
- The 8 month old mice were treated twice daily for a month with the PI3K inhibitor. Mice were then analyzed for presence of B cell subsets and plasma harvested to determine levels of anti-dsDNA antibodies. We noted that drug treatment markedly reduced percentage of Tfh cells (involved in stimulating B cell affinity maturation). In addition germinal center B cells (B cells undergoing affinity maturation—should represent the potentially pathogenic population)

were absent after treatment. Together this suggest that targeting the delta isoform of PI3K may be a useful therapeutic approach for lupus.

- Analysis of plasma has not been performed yet.

5. CONCLUSION: Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The first year of studies provided an initial window to examine the effect of targeting PI3Kdelta on lupus prone B cells and mice. Conclusions at this point remain preliminary.

We were surprised to see that only one month of treatment with drug had a dramatic effect on two key immune populations involved with producing pathogenic antibodies. The Tfh and germinal center B cells were highly reduced after treatment. This bodes well for expecting that the amount of IgG and anti-dsDNA antibodies will be reduced. Although the drug treatment affects B cells, only subsets of B cells were reduced. These tended to include subsets associated with being antigen specific, i.e. germinal center B cells and class switched B cells (have seen antigen). In contrast, naïve B cells appear unaffected. These would be key markers for demonstrating the therapeutic relevance of targeting PI3K in B cells. It is tempting to speculate that this treatment could be used to treat patients transiently, i.e. they would receive inhibitor for a month, then stay off the drug for 6-12 months, until the levels of lupus antibodies develop. This approach would be expected to minimize the immunocompromised state caused by drug treatment.

One goal of our future plans is to determine if the treatment extends the life on the NZB/NZW mice. Normally they have a shorted lifespan and often die from kidney damage. We have another cohort of mice that will examine this question. We are beginning to evaluate the minimum amount of time needed to treat with drug in order to reduce presence of the pathogenic immune populations.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:
(2) Peer-Reviewed Scientific Journals:
(3) Invited Articles:
(4) Abstracts:

Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8. REPORTABLE OUTCOMES:

Nothing to report

9. OTHER ACHIEVEMENTS:

Nothing to report

10. REFERENCES: n/a

11. APPENDICES: n/a